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New Quadriplex PCR Assay for Detection of Methicillin and Mupirocin Resistance and Simultaneous Discrimination of *Staphylococcus aureus* from Coagulase-Negative Staphylococci

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Major challenges in diagnostic molecular microbiology are to develop a simple assay to distinguish *Staphylococcus aureus* from the less virulent but clinically important coagulase-negative staphylococci (CoNS) and to simultaneously determine their antibiotic resistance profiles. Multiplex PCR assays have been developed for the detection of methicillin- and mupirocin-resistant *S. aureus* and CoNS but not for the simultaneous discrimination of *S. aureus* from CoNS. We designed a new set of *Staphylococcus* genus-specific primers and developed a novel quadriplex PCR assay targeting the 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), *mecA* (a determinant of methicillin resistance), and *mupA* (a determinant of mupirocin resistance) genes to identify most staphylococci, to discriminate *S. aureus* from CoNS and other bacteria, and to simultaneously detect methicillin and mupirocin resistance. Validation of the assay with 96 ATCC control strains and 323 previously characterized clinical isolates, including methicillin- and mupirocin-sensitive and -resistant *S. aureus* and CoNS isolates and other bacteria, demonstrated 100% sensitivity, specificity, and accuracy. This assay represents a simple, rapid, accurate, and reliable approach for the detection of methicillin- and mupirocin-resistant staphylococci and offers the hope of preventing their widespread dissemination through early and reliable detection.

Members of the genus *Staphylococcus* are major human pathogens, causing a wide variety of hospital- and community-acquired infections worldwide. *Staphylococcus aureus*, in particular, is a leading cause of diseases ranging from mild skin and soft tissue infections to life-threatening illnesses, such as deep postsurgical infections, septicemia, and toxic shock syndrome (9). Coagulase-negative staphylococci (CoNS) had been regarded as harmless skin commensals prior to the 1970s; however, they are now recognized as important causes of human infections (23, 36, 37). Data taken from the United States National Nosocomial Infections Surveillance System from January 1990 to May 1999 showed that CoNS were the most commonly reported pathogens (37.3% for CoNS versus 12.6% for *S. aureus*) isolated from bloodstream infections in intensive care unit patients (1). The importance of CoNS as major nosocomial pathogens in the context of prosthetic and indwelling device-related infections has been recognized (18). CoNS are also among the most frequently isolated bacteria in clinical microbiology laboratories (29, 31). More importantly, CoNS often serve as reservoirs of antimicrobial resistance determinants, since they usually have a high prevalence of multidrug resistance (5). Therefore, it is important to characterize and distinguish *S. aureus* strains and CoNS.

Both *S. aureus* and CoNS share a remarkable ability to accumulate additional antibiotic resistance determinants, resulting in the formation of multidrug-resistant strains. Such strains have demonstrated resistance to penicillins, clindamycin, tetracyclines, macrolides, linezolid, methicillin (MET) and, more recently, mupirocin (MUP) and vancomycin. This resistance limits therapeutic options for treatment and substantially increases patient morbidity and mortality.

The unique bactericidal action of MUP makes it one of the few antibiotics still effective against MET-resistant *S. aureus* (MRSA) and MET-resistant CoNS (MR-CoNS). MUP (pseudomonic acid A) is, in part, an analogue of isoleucine and exerts its antimicrobial effect by abolishing protein synthesis through irreversible and specific binding to bacterial isoleucyl-tRNA synthetase encoded by a chromosomal *ileS* gene (19). Topical MUP plays a crucial role in strategies designed to control outbreaks of MRSA and MR-CoNS, particularly in peritoneal dialysis, hemodialysis, and postsurgical patients (12). However, with the increasing use of MUP, resistance to it among staphylococci has emerged, threatening its clinical utility (6–8). Two MUP resistance phenotypes, low-level MUP resistance (L-MuR, with an MIC of 8 to 256 μg/ml) and high-

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TABLE 1. Phenotypic and genotypic features of *S. aureus* and CoNS control strains

Identification no.[e]	Strain	MIC (μg/ml) of[a]:		Positive (+) or negative (−) result for the following gene[b]:			
		OXA[c]	MUP[d]	16S rRNA	*mupA*	*mecA*	*nuc*
1	MSSA (ATCC 29213)	0.25	0.12	+	−	−	+
2	MUP-susceptible S. *aureus* (ATCC 25923)	0.25	0.25	+	−	−	+
3	MRSA (ATCC 33591)	>256	0.12	+	−	+	+
4	H-MuR-MRSA-80	>256	>1,024	+	+	+	+
5	H-MuR-MRSA-85	>256	>1,024	+	+	+	+
6	H-MuR-MRSA-221	>256	>1,024	+	+	+	+
7	L-MuR-MRSA-218	>256	16.0	+	−	+	+
8	L-MuR-MRSA-246	>256	12.0	+	−	+	+
9	H-MuR-MSSA-12	0.12	>1,024	+	+	−	+
10	H-MuR-MSSA-17	0.25	>1,024	+	+	−	+
11	H-MuR-MSSA-26	0.25	>1,024	+	+	−	+
12	H-MuR-MR-CoNS-P14	>256	>1,024	+	+	+	−
13	H-MuR-MS-CoNS-P15	0.19	>1,024	+	+	−	−
14	H-MuR-MS-CoNS-P16	0.19	>1,024	+	+	−	−
15	*S. epidermidis* (ATCC 14990)	0.12	0.12	+	−	−	−
16	*S. saprophyticus* (ATCC 13518)	1.5	4.0	+	−	−	−
17	*S. haemolyticus* (ATCC 29970)	0.19	0.38	+	−	−	−
18	*S. lugdunensis*	1.0	0.25	+	−	−	−
19	*S. sciuri* (ATCC 29060)	1.5	0.38	+	−	−	−
20	*S. simulans* (ATCC 27851)	0.38	0.19	+	−	−	−
21	*S. xylosus* (ATCC 29971)	1.0	0.5	+	−	−	−
22	*S. pyogenes* (ATCC 19615)	ND	ND	−	−	−	−

[a] The MIC was determined by the E-test according to the manufacturer's instructions. ND, not done.
[b] 16S rRNA, *Staphylococcus* genus specific; *mupA*, determinant of H-MuR; *mecA*, determinant of MET resistance; *nuc*, *S. aureus* species specific.
[c] For the OXA E-test, MICs of ≤2 μg/ml indicate susceptibility and MICs of ≥4 μg/ml indicate resistance in *S. aureus*, and MICs of ≤0.25 μg/ml indicate susceptibility and ≥0.5 μg/ml indicate resistance in CoNS; however, MICs of 0.5 to 2 μg/ml and negative results for the *mecA* gene were also recorded as susceptibility in CoNS (26).
[d] For the MUP E-test, MICs of ≤4 μg/ml indicate susceptibility, MICs of ≥512 μg/ml indicate H-MuR, and MICs of 8 to 256 μg/ml indicate L-MuR in both *S. aureus* and CoNS (2, 8, 10).
[e] Identification numbers in first column correspond to lane numbers in Fig. 2B.

level MUP resistance (H-MuR, with an MIC of ≥512 μg/ml), have been identified (8, 11, 15). L-MuR arises from point mutations within the host *ileS* gene (4, 8, 11, 38) and is of controversial clinical significance, while H-MuR results from the acquisition of a transferable plasmid containing the *mupA* (or *ileS-2*) gene, encoding an additional isoleucyl-tRNA synthetase that is not bound by MUP (17).

This worldwide resistance problem has made it crucial for clinical laboratories to implement a rapid, accurate, and simple method for the identification and discrimination of MRSA, MUP-resistant *S. aureus*, and CoNS. The use of PCR for the detection of *mecA* (encoding a low-affinity penicillin-binding protein [PBP2a] that confers broad resistance to all penicillin-related compounds) (16) and *mupA* (3, 14, 32, 38) was previously described. Only three previous reports described the simultaneous detection of *mecA* and *mupA* in *S. aureus* (28, 30) or in CoNS (13), and they did not describe the identification and discrimination of *S. aureus* and CoNS or the simultaneous detection of resistance genes in a single PCR.

Here we report a novel quadriplex PCR assay developed to identify most staphylococci, to discriminate *S. aureus* from CoNS and other bacteria, and to simultaneously detect MET resistance and MUP resistance.

MATERIALS AND METHODS

Bacterial strains and isolates. The bacterial control strains used in our study included a number of strains of *S. aureus*, CoNS, and various nonstaphylococci that were obtained from the American Type Culture Collection (ATCC, Manassas, Va.), as well as previously characterized clinical isolates of CoNS obtained from the Department of Microbiology and Infection Control, London Health Science Centre (LH), London, Ontario, Canada (20, 21). Clinical isolates of MUP-resistant *S. aureus* were obtained from the Toronto Medical Laboratory, Riverdale Hospital, Toronto, Ontario, Canada, and clinical isolates of MUP-resistant CoNS were obtained from Calgary Laboratory Services (CLS), Calgary, Alberta, Canada; these clinical isolates underwent subsequent phenotypic and genotypic characterizations at the Centre for Antimicrobial Resistance, Calgary, Alberta, Canada (Table 1). Clinical isolates used for validation of the multiplex PCR (M-PCR) assay were obtained from CLS during the period from August 1999 to May 2003 and from the Toronto Medical Laboratory during the period from January 1999 to April 2001.

Identification and phenotypic susceptibility testing of staphylococcal isolates. The staphylococcal isolates were identified morphologically and biochemically by standard laboratory procedures (25). The coagulase plasma test (Remel, Lenexa, Kans.) was performed on organisms exhibiting typical staphylococcal colony morphology to allow for discrimination of *S. aureus* from CoNS. Screening for MET resistance and other antibiotic resistance phenotypes was done by Vitek (bioMerieux, Inc., Durham, N.C.) or Kirby-Bauer methods, while confirmation of MET resistance was achieved with a routine PCR assay for the *mecA* gene (21). The MET and MUP MICs were further confirmed by the E-test according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). MUP sensitivity was defined as MICs of ≤4 μg/ml, H-MuR was defined as MICs of ≥512 μg/ml, and L-MuR was defined as MICs ranging from 8 to 256 μg/ml in both *S. aureus* and CoNS (2, 8, 10). MET susceptibility and MET resistance were defined as oxacillin (OXA) E-test MICs of ≤2 and ≥4 μg/ml, respectively, in *S. aureus* or MICs of ≤0.25 and ≥0.5 μg/ml, respectively, in CoNS. CoNS strains for which the OXA MIC was 0.5 to 2 μg/ml but which did not possess the *mecA* gene were recorded as MET susceptible according to NCCLS guidelines (26).

DNA extraction. Frozen bacteria were subcultured twice on 5% sheep blood Columbia agar plates (PML Microbiologicals, Wilsonville, Oreg.) prior to DNA extraction. For rapid DNA extraction, about one to five bacterial colonies were suspended in 50 μl of sterile distilled water and heated at 99°C for 10 min. After centrifugation at 20,000 × *g* for 1 min, 5 μl of the supernatant was used as a template in a 25-μl PCR mixture.

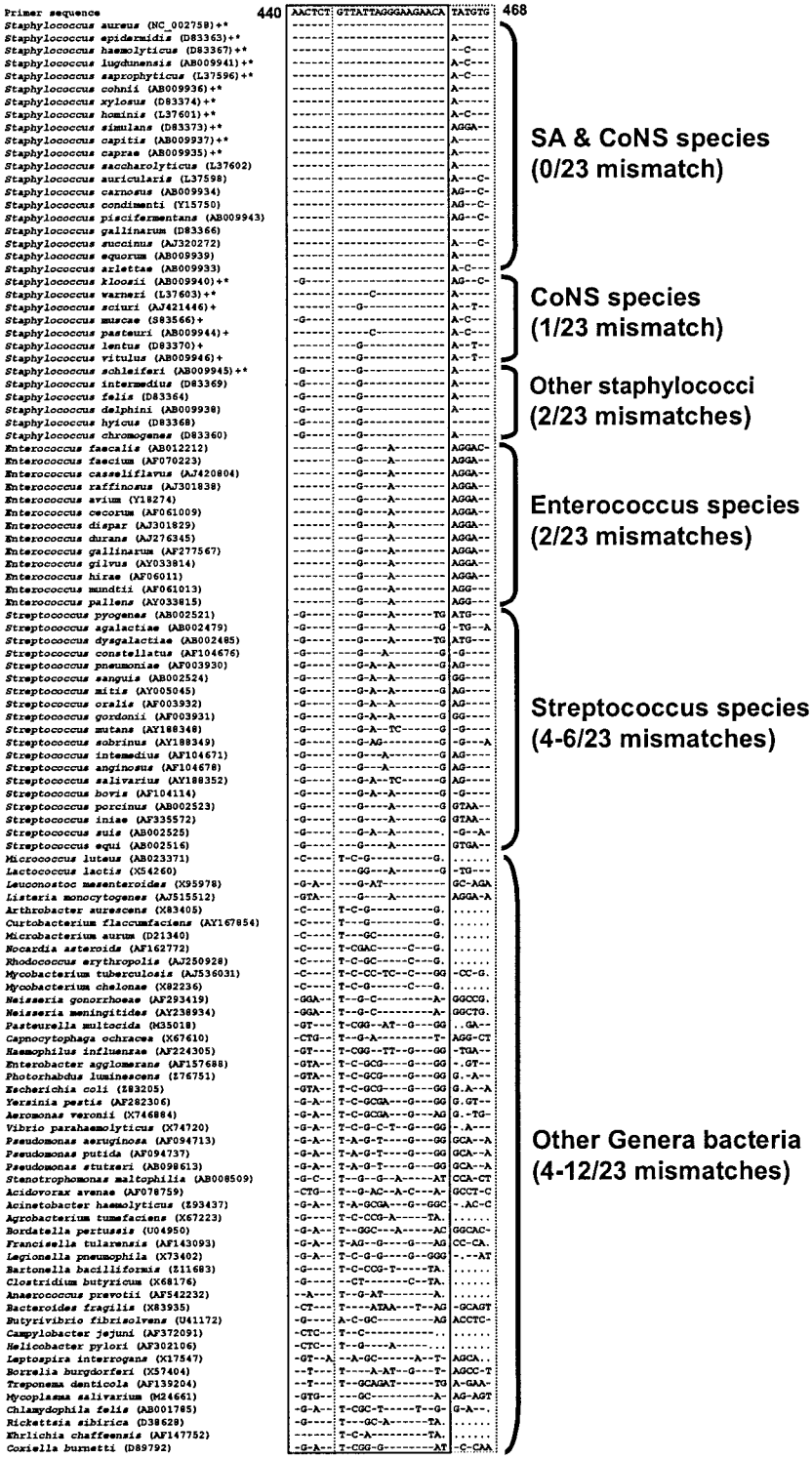

FIG. 1. Comparison of nucleotide sequences targeted by our *Staphylococcus* genus-specific 16S rRNA 5′ primer (Staph756F) and a previously published primer for various representative species of staphylococci and nonstaphylococci. The primer sequence is shown at the top with *S. aureus* strain Wu50 numbering. The solid and dotted boxes indicate the sequences of our primer (Staph756F) and the previously published primer (22, 24), respectively. Bacterial 16S rRNA gene sequences were obtained from the GenBank database; nucleotide sequence accession numbers are shown in parentheses. Staphylococcal species indigenous to human beings are denoted by plus signs, and medically important species are denoted by asterisks. The numbers of nucleotide mismatches with our new primer are shown on the right. SA, *S. aureus*.

16S rRNA gene sequence alignment and oligonucleotide primers. With Lasergene Expert sequence analysis software (DNASTAR, Inc., Madison, Wis.) and the GenBank database (National Center for Biotechnology Information), an extensive and comprehensive bacterial 16S rRNA gene sequence database was constructed. This database contained approximately 5,500 complete 16S rRNA gene sequences currently available in the GenBank database (updated as of May 2003) and representing all clinically significant bacteria (species and strains) (25), including 19 sequences from various strains of *S. aureus*, 78 sequences from various strains of 32 species of CoNS, 77 sequences from various strains of 13 species of *Enterococcus*, 226 sequences from various strains of 19 species of *Streptococcus*, and 5,100 sequences from other bacteria. A new *Staphylococcus* genus-specific 5′ primer (Staph756F) was designed based on the comprehensive alignment of the bacterial 16S rRNA gene sequences.

The oligonucleotide primers used in this study were synthesized and purchased from Qiagen Operon (Qiagen, Inc., Alameda, Calif.). The primers included the newly designed primer Staph756F (5′-AAC TCT GTT ATT AGG GAA GAA CA-3′) and the previously published 3′ primer Staph750R (5′-CCA CCT TCC TCC GGT TTG TCA CC-3′) (22) for *Staphylococcus* genus-specific 16S rRNA, Nuc 1 (5′-GCG ATT GAT GGT GAT ACG GTT-3′) and Nuc 2 (5′-AGC CAA GCC TTG ACG AAC TAA AGC-3′) for *nuc* (34), MupA (5′-TAT ATT ATG CGA TGG AAG GTT GG-3′) and MupB (5′-AAT AAA ATC AGC TGG AAA GTG TTG-3′) for *mupA* (3), and MecA1 (5′-GTA GAA ATG ACT GAA CGT CCG ATA A-3′) and MecA2 (5′-CCA ATT CCA CAT TGT TTC GGT CTA A-3′) for *mecA* (33).

PCR amplification. A quadriplex PCR assay targeting 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), *mupA* (a determinant of MUP resistance), and *mecA* (a determinant of MET resistance) was developed and used in our study. M-PCR assays were regularly performed directly with bacterial suspensions obtained after rapid DNA extraction. An aliquot of 5 μl of bacterial suspension was added to a 20-μl PCR mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dATP, dUTP, dGTP, and dCTP) (Invitrogen, Inc., Carlsbad, Calif.), 0.12 μM each 16S rRNA and *mecA* primers, 0.05 μM each *mupA* primer, 0.04 μM each *nuc* primer, and 1.0 U of Platinum *Taq* DNA polymerase (Invitrogen). Amplification was performed by using a GeneAmp PCR system 9700 or 9600 thermal cycler (Applied Biosystems, Foster City, Calif.). Amplification was carried out as follows: an initial denaturation step at 94°C for 5 min; 10 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. For increased specificity, strict PCR conditions were used as follows: an initial denaturation step at 94°C for 5 min; 10 cycles of 94°C for 40 s, 68°C for 40 s, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. For single-target amplification, PCR was carried out with a 20-μl PCR mixture containing 0.2 μM each primer and with the following cycling parameters: an initial denaturation step at 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. All M-PCR assay runs incorporated a reagent control (without template DNA) and a true-negative control (*Streptococcus pyogenes* ATCC 19615) processed in a fashion similar to that of the clinical isolates. The PCR amplicons were visualized by using a UV light box after electrophoresis on a 2% agarose gel containing 0.5 μg of ethidium bromide/ml.

Limiting-dilution experiments for estimation of M-PCR sensitivity. The sensitivity of M-PCR amplification with various pairs of primers was estimated by limiting-dilution experiments. Briefly, bacterial cultures from overnight growth at 37°C on a blood agar plate were suspended in sterile saline (Vitek saline) to a density corresponding to a 1.0 McFarland turbidity standard by using a Vitek colorimeter (bioMerieux). These suspensions were used to prepare serial 10-fold dilutions with sterile double-distilled water. DNA extraction by the rapid method described above was performed for 50 μl of each dilution. The standard M-PCR assay was performed to determine its sensitivity. The lower limits of detection (or the minimal numbers of CFU detectable) of the target genes by M-PCR were calculated based on correlation of the 1.0 McFarland standard to 3×10^8 CFU/ml.

Double-blind validation of M-PCR. Validation of M-PCR was conducted in a double-blind fashion. A technical staff person who knew the phenotypic microbiological data, including the free coagulase plasma test results and the OXA and MUP susceptibility results for the tested isolates, assigned the isolate numbers. Another technologist was involved in subculturing the strains and performing the PCRs but was unaware of the phenotypic microbiological test results. The results were compared when the tests were completed.

RESULTS

Identification of novel *Staphylococcus* genus-specific primers. The sequences of the previously published *Staphylococcus* genus-specific primers (22, 24) were not well conserved in the *Staphylococcus* genus (Fig. 1). To design new *Staphylococcus* genus-specific primers, we constructed an extensive and comprehensive bacterial 16S rRNA gene sequence database. A new *Staphylococcus* genus-specific 5′ primer (Staph756F) was designed based on the comprehensive alignment of the bacterial 16S rRNA gene sequences. This novel primer targets the 16S rRNA gene (nucleotides 440 to 462 of the *S. aureus* strain Wu50 16S rRNA gene) and is *Staphylococcus* genus specific and well conserved in the sequences of almost all of the *Staphylococcus* species and strains currently available in the GenBank database. Figure 1 shows a comparison of the nucleotide sequences targeted by our Staph756F primer and the previously published *Staphylococcus* 16S rRNA gene 5′ primer for various representative species of staphylococci, enterococci, streptococci, and other bacterial genera. Our new primer was more conserved and genus specific than the previously published primer, with perfect nucleotide sequence matches in all 19 sequences of *S. aureus* examined and the most medically important CoNS species, including *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus*, *S. cohnii*, *S. xylosus*, *S. hominis*, *S. simulans*, *S. capitis*, and *S. caprae*; there was only one base mismatch out of 23 nucleotides in the other seven CoNS species of human or animal origin (*S. kloosii*, *S. warneri*, *S. sciuri*, *S. muscae*, *S. pasteuri*, *S. lentus*, and *S. vitulus*), but there were two mismatches in one medically important staphlococcal species (*S. schleiferi*) (Fig. 1). A combination of the new 5′ primer with the previously published 3′ primer for *Staphylococcus* 16S rRNA (Staph750R) (22, 24) in our novel quadriplex PCR assay allowed us to specifically detect most species and strains of staphylococci.

New quadriplex PCR for identifying staphylococci, discriminating *S. aureus* from CoNS and other bacteria, and simultaneously detecting H-MuR and MET resistance. We developed a new M-PCR assay to discriminate *S. aureus* from CoNS and other bacteria and to simultaneously detect MET resistance and MUP resistance. The quadriplex PCR assay targeted the 16S rRNA, *nuc*, *mupA*, and *mecA* genes. To ensure that each individual primer pair was adequate for the amplification of all four gene fragments, the single-target PCR protocol with each individual primer pair was conducted prior to M-PCR optimization with our control strains (Table 1). The individual amplifications yielded the fragments of the expected sizes, i.e., 756, 457, 310, and 279 bp, for the 16S rRNA, *mupA*, *mecA*, and *nuc* genes, respectively (Fig. 2A). M-PCR optimization was obtained by assaying different primer concentrations and other PCR components. Amplification of 16S rRNA, *mupA*, *mecA*, and *nuc* targets of phenotypically and genotypically characterized control strains, including groups of MET- and MUP-sensitive or -resistant *S. aureus* strains and CoNS or other bacteria (Table 1), in a single M-PCR produced distinct bands corresponding to respective molecular sizes that were easily recognizable in agarose gels stained with ethidium bromide (Fig. 2B).

Specificity of M-PCR. Although the new Staph756F primer was found to be well conserved in all *S. aureus* strains and most

TABLE 2. M-PCR specificity in various species and strains of staphylococci and other nonstaphylococcal bacteria

Species or subspecies	Strain	Positive (+) or negative (−) M-PCR result for the following gene:			
		16S rRNA	*mupA*	*mecA*	*nuc*
Staphylococcus auricularis	LH 8972	+	−	−	−
	LH 9747	+	−	−	−
Staphylococcus capitis subsp. *capitis*	LH 3052	+	−	−	−
	LH 4984	+	−	−	−
Staphylococcus capitis subsp. *ureolyticus*	LH 2446	+	−	−	−
	LH 2619	+	−	−	−
Staphylococcus caprae	LH 2455	+	−	−	−
	LH 2589	+	−	−	−
Staphylococcus cohnii subsp. *cohnii*	LH 9675	+	−	−	−
	LH 9691	+	−	−	−
Staphylococcus cohnii subsp. *urealyticum*	LH 4518	+	−	−	−
	LH 4992	+	−	−	−
Staphylococcus epidermidis	ATCC 14990	+	−	−	−
	ATCC 12228	+	−	−	−
	LH 2575	+	−	+	−
	LH 2617	+	−	−	−
Staphylococcus haemolyticus	ATCC 29970	+	−	−	−
	LH 2442	+	−	+	−
	LH 2583	+	−	+	−
Staphylococcus hominis	LH 2356	+	−	+	−
	LH 3077	+	−	+	−
Staphylococcus intermedius	LH 4230	+	−	−	−
	LH 4234	+	−	−	−
Staphylococcus lentus	LH 4725	+	−	−	−
	LH 4879	+	−	−	−
Staphylococcus lugdunensis	LH 2398	+	−	−	−
	LH 3143	+	−	−	−
	CLS-Patient	+	−	−	−
Staphylococcus saprophyticus	ATCC 13518	+	−	−	−
	ATCC 15305	+	−	−	−
	LH 2306	+	−	−	−
	LH 3069	+	−	−	−
Staphylococcus schleiferi subsp. *schleiferi*	LH 2352	+	−	−	−
	LH 2440	+	−	−	−
Staphylococcus schleiferi subsp. *coagulans*	LH 4885	+	−	−	−
	LH 4886	+	−	−	−
Staphylococcus sciuri	ATCC 29060	+	−	−	−
	LH 3744	+	−	−	−
	LH 3780	+	−	−	−
Staphylococcus simulans	ATCC 27851	+	−	−	−
	LH 3118	+	−	−	−
	LH 3238	+	−	−	−
Staphylococcus warneri	LH 2852	+	−	−	−
	LH 2967	+	−	−	−
Staphylococcus xylosus	ATCC 29971	+	−	−	−
	LH 2136	+	−	−	−
	LH 4892	+	−	−	−
Nonstaphylococci[a]		−	−	−	−

[a] Thirty-six strains, including *Streptococcus pyogenes* (ATCC 19615), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus pneumoniae* (ATCC 6303), *Streptococcus sanguis* (ATCC 10556), *Streptococcus constellatus* (CLS isolate), *Streptococcus anginosus* (CLS isolate), *Streptococcus intermedius* (CLS isolate), *Enterococcus avium* (ATCC 14025), *Enterococcus casseliflavus* (7505-97), *Enterococcus durans* (ATCC 19432 and ATCC 6056), *Enterococcus faecalis* (ATCC 19433, ATCC 29212, ATCC 33186, ATCC 59532, ATCC 51299, and N00410), *Enterococcus faecium* (ATCC 35667, ATCC 49032, N97–330, and CLS isolate), *Enterococcus gallinarum* (ATCC 49573), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Klebsiella oxytoca* (CLS isolate), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853), *Serratia marcescens* (ATCC 8100), *Enterobacter cloacae* (ATCC 13047), *Citrobacter freundii* (ATCC 8090), *Morganella morganii* (CLS isolate), *Providencia stuartii* (ATCC 33672), *Leuconostoc mesenteroides* (ATCC 8293), *Micrococcus luteus* (ATCC 533), *Bacillus cereus* (ATCC 14579), and *Aerococcus viridans* (ATCC 11563).

TABLE 3. M-PCR sensitivity in various representative strains

Strain	Sensitivity (CFU/PCR) for the following gene:[a]			
	16S rRNA	*mupA*	*mecA*	*nuc*
MSSA (ATCC 29213)	3×10^5	NA	NA	3×10^5
MRSA (ATCC 33591)	3×10^5	NA	1.5×10^6	3×10^5
H-MuR-MRSA-221	3×10^5	1.5×10^6	1.5×10^6	3×10^5
L-MuR-MRSA-218	3×10^5	NA	3×10^5	3×10^5
H-MuR-MSSA-12	3×10^5	1.5×10^6	NA	3×10^5
S. epidermidis (ATCC 14990)	1.5×10^6	NA	NA	NA
S. saprophyticus (ATCC 13518)	1.5×10^6	NA	NA	NA
S. sciuri (ATCC 29060)	1.5×10^6	NA	NA	NA
H-MuR-MR-CoNS-P14	1.5×10^6	1.5×10^6	1.5×10^6	NA
H-MuR-MS-CoNS-P16	3×10^5	1.5×10^6	NA	NA

[a] NA, not applicable (minimal number of CFU/PCR).

TABLE 4. Validation of M-PCR assay in clinical isolates[a]

Organism	Group	No. of isolates	Positive (+) or negative (−) M-PCR result for the following gene:			
			16S rRNA	*mupA*	*mecA*	*nuc*
S. aureus	H-MuR MRSA	56	+	+	+	+
	H-MuR MSSA	6	+	+	−	+
	L-MuR MRSA	6	+	−	+	+
	MUP-susceptible MRSA	49	+	−	+	+
	MUP-susceptible MSSA	47	+	−	−	+
CoNS	H-MuR MR-CoNS	72	+	+	+	−
	H-MuR MS-CoNS	35	+	+	−	−
	L-MuR MR-CoNS	1	+	−	+	−
	L-MuR MS-CoNS	1	+	−	−	−
	MUP-susceptible MR-CoNS	19	+	−	+	−
	MUP-susceptible MS-CoNS	31	+	−	−	−

[a] The correlation for all results was 100%.

strains and species of CoNS, one or two nucleotide mismatches were found in several non-medically important species of staphylococci as well as in some members of the genus *Enterococcus* (Fig. 1). To address the specificity of M-PCR, we tested 47 staphylococcal strains from 19 species or subspecies and 36 nonstaphylococcal bacteria from 27 species of 15 genera, including 15 *Enterococcus* strains belonging to 6 medically important species (Table 2). The novel M-PCR detected all species of CoNS tested and did not cross-react with other bacteria (Table 2), except for several *Enterococcus* strains tested under general M-PCR conditions. For a total of 15 *Enterococcus* strains tested (Table 2), no cross-reaction was observed with 2 strains (*Enterococcus faecium* ATCC 35667 and ATCC 49032), while faint visible bands were seen in 5 strains (*E. casseliflavus* 7505-97; *E. faecalis* ATCC 59532, ATCC 51299, and N00410; and *E. faecium* CLS isolate) and almost imperceptible bands were seen in the remaining 8 strains (*E. avium* ATCC 14025; *E. durans* ATCC 19432 and ATCC 6056; *E. faecalis* ATCC 19433, ATCC 2912, and ATCC 33186; *E. faecium* N97-330; and *E. gallinarum* ATCC 49573). However, these faint cross-reactive 16S rRNA gene bands in enterococcal bacteria disappeared under strict M-PCR conditions (data not shown).

Sensitivity of M-PCR. The sensitivity of our M-PCR assay was examined with 10 representative strains and species of staphylococcal bacteria, including MET-susceptible *S. aureus* (MSSA), MRSA, H-MuR MRSA, L-MuR MRSA, H-MuR MSSA, H-MuR MR-CoNS, and H-MuR MET-susceptible CoNS (MS-CoNS), as well as 2 species (*S. epidermidis* and *S. saprophyticus*) of CoNS with sequences perfectly matching the sequence of our Staph756F primer and a species (*S. sciuri*) with one nucleotide mismatch (Fig. 1) (Table 3). Under general M-PCR conditions, the assay was capable of detecting, with reproducibility, bands in ethidium bromide-stained gels at dilutions corresponding to 3.0×10^5 to 1.5×10^6 CFU/PCR; the values varied slightly depending on the strain and specific gene examined (Table 3). Overall, the single-target PCR was only 2 to 10 times (1.5×10^5 to 1.5×10^6) more sensitive than our M-PCR (data not shown), suggesting that our M-PCR assay is sufficiently robust.

Validation of M-PCR. Validation of our quadriplex assay was performed in a double-blind fashion. The results of PCR testing of 323 previously well-characterized clinical isolates, including H-MuR MRSA ($n = 56$), H-MuR MSSA ($n = 6$), L-MuR MRSA ($n = 6$), MUP-susceptible MRSA ($n = 49$), MUP-susceptible MSSA ($n = 47$), H-MuR MR-CoNS ($n = 72$), H-MuR MS-CoNS ($n = 35$), L-MuR MR-CoNS ($n = 1$), MUP-resistant MS-CoNS ($n = 1$), MUP-susceptible MR-CoNS ($n = 19$), and MUP-susceptible MS-CoNS ($n = 31$) (Table 4), were compared to the results of staphylococcal identification by a free coagulase plasma test and OXA and MUP E-test susceptibility results. We found a 100% concordance between microbiological and PCR results, including a correlation with antibiotic susceptibility phenotypes (Table 4).

DISCUSSION

S. aureus and CoNS are major causes of community-acquired and nosocomial infections (1, 9, 18, 27, 36). They are also among the most frequently isolated bacteria in clinical microbiology laboratories (29, 31). Simple and rapid identification and discrimination of *S. aureus* and CoNS and detection of MET resistance and MUP resistance are essential for prompt institution of effective antimicrobial chemotherapy and for limiting the unnecessary use of certain classes of antibiotics. Since MET resistance (encoded by *mecA*) and H-MuR (encoded by *mupA*) are mediated by the expression of PBP2a and by the expression of altered isoleucyl-tRNA synthetase, respectively, PCR detection of *mecA* and *mupA* is considered the "gold standard" for the detection of MET resistance and H-MuR (3, 14, 26). Although previous reports evidenced the utility of PCR for the accurate detection of the *mupA* gene (3, 14, 32, 38), only two studies have included the simultaneous detection of the *mecA* and *mupA* genes (28, 30). Nunes et al. (28) were the first to attempt to include *femA* (an auxiliary gene found in all *S. aureus* strains), *mecA*, and *mupA* in an M-PCR to allow for the identification of *S. aureus* and the simultaneous detection of MET resistance and MUP resistance in a single PCR. However, these investigators failed to amplify all three targets in a single-tube reaction due to technical problems and finally had to perform two separate PCRs for the detection of *mecA* and *mupA* in one tube and *femA* in another tube (28). Ferreira et al. applied the same duplex PCR for the detection of the *mecA* and *mupA* genes in CoNS but did not include any specific bacterial identification marker genes (13). Recently, Perez-Roth et al. (30) demonstrated the feasi-

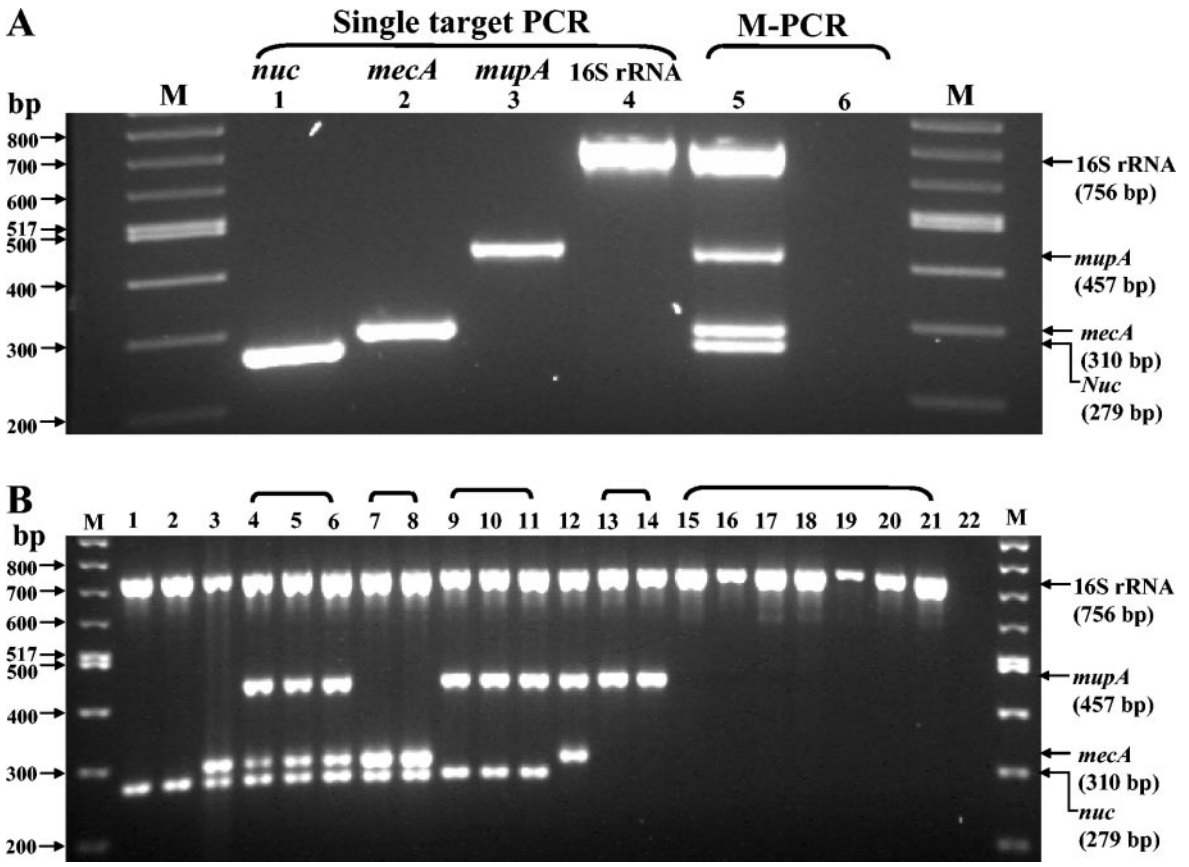

FIG. 2. M-PCR identifies all staphylococci, discriminates *S. aureus* from CoNS and other bacteria, and simultaneously detects H-MuR and MET resistance. (A) Single-target PCR and M-PCR products of the 16S rRNA, *mupA*, *mecA*, and *nuc* genes amplified from control strain H-MuR-MRSA-85. Lanes 1 to 4, single-target PCR amplicons obtained from the *nuc*, *mecA*, *mupA*, and 16S rRNA genes with the corresponding single primers, respectively. Lane 5, quadriplex PCR amplicons from the 16S rRNA, *mupA*, *mecA*, and *nuc* genes simultaneously amplified. Lane 6, negative control for M-PCR in lane 5. (B) M-PCR detecting the 16S rRNA, *mupA*, *mecA*, and *nuc* genes in control strains for MSSA (lane 1), MUP-susceptible *S. aureus* (lane 2), MRSA (lane 3), H-MuR MRSA (lanes 4 to 6), L-MuR MRSA (lanes 7 and 8), H-MuR MSSA (lanes 9 to 11), H-MuR MR-CoNS (lane 12), H-MuR MS-CoNS (lanes 13 and 14), various species of CoNS (lanes 15 to 21), and a non-*S. aureus* non-CoNS control strain (lane 22). Table 1 provides details for the strains. Lanes M in both panel A and panel B, molecular size marker (100-bp DNA ladder; New England BioLabs).

bility of an M-PCR for the simultaneous identification of *S. aureus* (*femB*) and detection of MET resistance and MUP resistance (*mecA* and *mupA*, respectively) but not for discrimination between *S. aureus* and CoNS.

We designed a novel set of *Staphylococcus* genus-specific primers based on a comprehensive alignment of bacterial 16S rRNA gene sequences and successfully developed a quadriplex PCR assay that targets 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), *mupA* (a determinant of MUP resistance), and *mecA* (a determinant of MET resistance) and that could identify most staphylococci, discriminate *S. aureus* from CoNS and other bacteria, and simultaneously detect MUP resistance and MET resistance. This assay, including our simple and rapid boiling method of DNA extraction, offers obvious advantages in clinical laboratory diagnosis and epidemiological surveillance programs.

The genus *Staphylococcus* is comprised of at least 35 unique species, with *S. aureus* and certain CoNS species being considered of great importance in terms of human health. Of the 32 CoNS species, only half have been recovered from humans (23, 36). The main medically important CoNS (most frequently reported species involved in human infections) are *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus*, *S. schleiferi*, *S. xylosus*, *S. hominis*, *S. simulans*, *S. cohnii*, *S. capitis*, *S. caprae*, *S. kloosii*, and *S. warneri*, although *S. epidermidis* accounts for most CoNS infections (23, 36, 37). Other staphylococcal species are of minor medical importance (indigenous to humans but less frequently reported as etiologic agents of human infections) or are not medically important (indigenous to other animals and birds and/or found occasionally as part of the transient human flora but without causing human infections) (25). These staphylococcal species and strains are phylogenetically diverse (35), and it is very difficult to select a genus-conserved genetic region for the design of a PCR primer. Previously published *Staphylococcus* genus-specific primers (22, 24) do not target completely conserved regions in the staphylococcal 16S rRNA gene, having about 1 to 4 nucleotide mismatches even in most medically important CoNS species, including *S. epidermidis* (1 of 23 nucleotide mismatches), *S. haemolyticus* (1 of 23 mismatches), *S. lugdunensis* (2 of 23 mismatches), *S. saprophyticus* (2 of 23 mismatches), *S. hominis* (2 of 23 mismatches), and *S. simulans* (4 of 23 mismatches) in

multiple-sequence alignments (Fig. 1). When these primers were used in our PCR assay, we were unable to detect important CoNS, such as *S. lugdunensis*, *S. saprophyticus*, *S. schleiferi*, *S. hominis*, *S. simulans*, and *S. sciuri* (data not shown).

We designed a new conserved *Staphylococcus* genus-specific 5′ primer (Staph756F) and integrated it into our new quadriplex PCR assay, allowing us to detect most species and strains of staphylococcal bacteria (Table 2). However, one or two nucleotide mismatches were noted in several species of nonmedically important staphylococci as well as in *Enterococcus* strains (Fig. 1). To address the cross-reactivity of staphylococci with enterococci in our M-PCR assay, we designed two sets of PCR conditions, general and strict (detailed in Materials and Methods). Under general conditions, the novel M-PCR detected all tested species and strains of *S. aureus* and CoNS; however, it resulted in faint, presumably cross-reactive bands for the 16S rRNA genes in several *Enterococcus* strains, but no cross-reactivity with other bacteria was seen. Under strict conditions, our assay specifically detected only staphylococci (Table 2); however, detailed sequence analysis predicts that several staphylococcal species, such as *S. schleiferi*, *S. intermedius*, *S. felis*, *S. delphini*, *S. hyicus*, and *S. chromogenes*, will be missed (Fig. 1). However, these staphylococcal species, with the exception of *S. schleiferi*, are not indigenous to human beings and are of no or minor medical importance (23, 36, 37). Therefore, different (general or strict) PCR conditions can be used, depending on the particular need, such as using general PCR conditions to detect all staphylococci but applying strict conditions when specificity is needed to exclude enterococci.

One of the limitations of our quadriplex assay is in the setting of mixed cultures of *S. aureus* and CoNS. As both species may carry *mecA* and/or *mupA*, PCR detection of these genes would indicate that one or both organisms harbor one or both of these genes. However, this situation is not expected to be a common occurrence, as organism purity should be ensured prior to PCR testing. Another limitation is the possibility of additional cross-reactions with other, rarely encountered species of medical significance. We were unable to test all species and acknowledge that as new species are identified, the possibility of cross-reactive bands for 16S rRNA genes exists, although our findings suggest that with the use of strict PCR conditions, this possibility would be a minor concern.

The current assay could detect only H-MuR and not L-MuR, since they result from different mechanisms. H-MuR results from the acquisition of a transferable plasmid containing the *mupA* (or *ileS-2*) gene, which is distinct from the chromosomal native *ileS* gene, sharing only 30% homology in the expected amino acid sequences (17). The genetic mechanism responsible for L-MuR is believed to be related to point mutations within the host *ileS* gene (4, 8, 11, 38), although the association between mutations and L-MuR is still not clear. Additional studies are needed to explore the genetic mechanism responsible for L-MuR in order to develop a comprehensive molecular diagnostic test that reliably detects all types of MUP resistance, including H-MuR and L-MuR.

In conclusion, we successfully developed a novel quadriplex PCR assay which could identify all staphylococci tested, discriminate *S. aureus* from CoNS and other bacteria, and simultaneously detect MUP resistance and MET resistance. This assay offers clinical laboratories a new rapid, simple, feasible, specific, and accurate tool. Its application will allow the rapid determination of MET resistance and MUP resistance in settings where it is used and hopefully will prevent the widespread dissemination of MET resistance and MUP resistance through early and reliable detection.

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